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# Induced pluripotent stem cells: epigenetic memories and practical implications

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**ABSTRACT:** Induced pluripotent stem cells (iPSCs) may be obtained by direct reprogramming of different somatic cells to a pluripotent state by forced expression of a handful of transcription factors. It was generally assumed that iPSCs are functionally equivalent to their embryonic stem cell (ESC) counterparts. Recently, a number of research groups have demonstrated that this is not the case, showing that iPSCs retain 'epigenetic memory' of the donor tissue from which they were derived and display skewed differentiation potential. This raises the question whether such cells are fit for experimental, diagnostic or therapeutic purpose. A brief survey of the literature illustrates that differences at both epigenetic and transcriptome level are observed between various pluripotent stem cell populations. Interestingly, iPSC populations with perceived 'anomalies' can be coaxed to a more ESC-like cellular state either by continuous passaging—which attenuates these epigenetic differences—or treatment with small molecules that target the machinery responsible for remodelling the genome. This suggests that the establishment of an epigenetic status approximating an ESC counterpart is largely a passive process. The mechanisms responsible remain to be established. Meanwhile, other areas of reprogramming are rapidly evolving such as, trans-differentiation of one somatic cell type to another by the forced expression of key transcription factors. When it comes to assessing their practical usefulness, the same question will also apply.

**Key words:** stem cells / epigenetics

## The current state of play . . .

It is generally agreed that pluripotent stem cells (PSCs) have huge experimental, diagnostic and therapeutic potential. However, it is unclear if inherent differences in the properties of PSCs obtained from different sources or by different means impact their applicability. The ability to revert a somatic cell to a pluripotent status has been achieved in a number of ways, including cell fusion and somatic-cell nuclear transfer (SCNT) (Gurdon, 1962; Tada *et al.*, 1997; Wilmot *et al.*, 1997; Tada *et al.*, 2001; Hochedlinger and Jaenisch, 2002). Recently, it was demonstrated that a somatic cell could be re-assigned to an embryonic-like state via the forced expression of a handful of key genes, notably Oct4, Sox2, Klf4 and c-Myc, to an induced pluripotent stem cell (iPSC) (Takahashi and Yamanaka, 2006). This technology is now in use in many laboratories worldwide and the lines produced are generally assumed to be functionally equivalent to embryonic stem cells (ESCs) derived from fertilized embryos (fESCs) or ESCs generated via SCNT (NT-ESCs). This is assessed by their appearance, expression of pluripotency markers, ability to form teratomas and, in the case of murine ESCs, the ability to generate chimeras or mice derived completely from iPSCs through tetraploid complementation.

Recently, a number of publications have questioned this, suggesting that iPSCs, fESCs and NT-ESCs are not equivalent and that iPSCs retain 'epigenetic memory' of the tissue from which they were derived (Marchetto *et al.*, 2009; Ji *et al.*, 2010; Kim *et al.*, 2010).

## Epigenetic differences and differentiation potential of iPSCs and ESCs

Kim *et al.* (2010) examined the impact of reprogramming murine somatic cell types (fibroblasts and blood) via 'somatic cell nuclear transfer' (SCNT) or forced expression of transcription factors on epigenetic parameters and ability to differentiate towards haematopoietic or osteogenic lineages compared with ESCs derived from a fertilized oocyte (fESCs). Strikingly, early passage iPSCs generated via forced expression of transcription factors exhibited vestiges of the methylation signature of the donor tissue from which they were derived, while the NT-ESCs exhibited an epigenetic profile very similar to fESCs. This was taken further by investigating the differentiation potential of iPSCs derived from either blood or fibroblasts. Again, a

'memory' was observed as the differentiation potential favoured the donor tissue from which the iPSCs were originally derived. In a related study, [Polo et al. \(2010\)](#) derived murine iPSCs from a number of different tissues, fibroblasts, granulocytes, lymphocytes (haematopoietic) and muscle (myogenic) cells. In agreement with [Kim et al. \(2010\)](#), early-passage iPSCs appeared to retain an epigenetic memory of the donor cell of origin and again this was reflected in differential gene expression and altered differentiation capacity. It is important to emphasize that these two studies were conducted in murine systems and their relevance to human iPSCs (hiPSCs) is uncertain. However, [Marchetto et al. \(2009\)](#) showed that hiPSCs derived from neural stem cells (NSC) were generally very similar to hESCs but the transcriptional profiles generated appeared to reveal a gene expression signature of the donor NSCs. It should be noted that this study was only undertaken in a single NSC line and requires further validation. However, these studies indicate that iPSCs potentially retain vestigial epigenetic characteristics consistent with the tissue from which they are derived ([Marchetto et al., 2009](#); [Kim et al., 2010](#); [Polo et al., 2010](#)).

The above studies complement a related study by [Ji et al. \(2010\)](#), who investigated whether specific DNA methylation marks could play a role in the regulation of developmental progression to a particular cell lineage. This was assessed by subjecting eight distinct populations of purified cells of the haematopoietic system to comprehensive high-throughput array-based relative methylation analysis (CHARM). This method determines the CpG methylation status of both promoters and nearby CpG shores at a genome-wide level. The outcome of this study demonstrated that lineage fate during differentiation in terms of commitment to myeloid or lymphoid development is potentially choreographed by modulation of the levels of DNA methylation.

It is clear from the above studies that there are differences at the molecular level between iPSCs and ESCs. However, if we look at mouse and human ESCs and iPSCs at a gross level, they share many features, including indefinite self-renewal and pluripotency in terms of the expression of pluripotency gene networks, the ability to differentiate two different tissue lineages via directed procedures, embryoid body formation or teratoma and, in the murine situation, the ability to form a live animal ([Carpenter et al., 2003](#); [Rao 2004](#); [Okita et al., 2007](#); [Smith et al., 2009](#)). But if one scrutinizes these model systems more closely, they are more divergent and certainly not equivalent. For example, mouse ESCs require leukaemia inhibitory factor (LIF) and bone morphogenic protein 4 (BMP4), while hESCs require activin and fibroblast growth factor 2 (FGF2) signalling ([Brons et al., 2007](#)). This demonstrates that these two cell types are indeed not equivalent at the molecular level but are still pluripotent. Interestingly, recent studies have demonstrated that mouse epiblast stem cells (EpiSC), derived from mouse embryos at post-implantation stage, are functionally closer to hESCs as they require activin and FGF2 signalling ([Vallier et al., 2007, 2009](#)).

There is other evidence that subtle differences exist between ESCs and iPSCs. A study by [Malchenko et al. \(2010\)](#) utilizing global comparative analysis of microRNAs (miRs) identified a number of miRs that were highly expressed in iPSCs, suggesting that these miRs were associated with cancers. This study was limited to interrogating two iPSCs lines and four hESC lines. Two other studies by [Chin et al. \(2009\)](#) and [Wilson et al. \(2009\)](#) noted that a subset of miRs were

consistently present in hiPSCs compared with hESCs, indicating that iPSCs may have a unique miR profile.

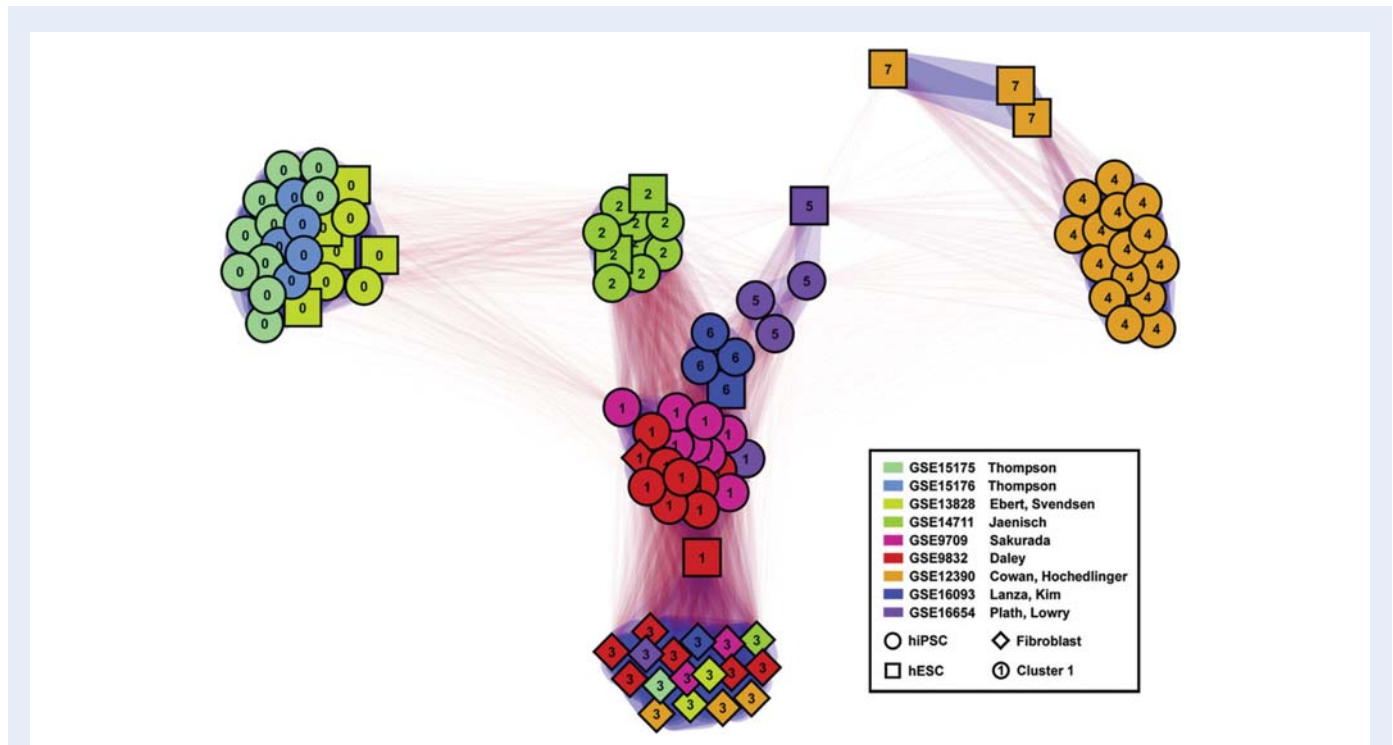
## Aberrant epigenetic profiles can be changed . . .

In an elegant study by [Stadtfield et al. \(2010\)](#), iPSCs versus ESCs were compared; note that in this study the pluripotent cells generated were genetically identical. The data demonstrated that overall the two cell types at the mRNA and miR level were almost identical, with the exception of a few transcripts. These differences were mapped to an imprinted gene cluster in the mouse genome on chromosome 12qF1.

Interestingly, two classes of iPSCs were observed. Those with aberrant silencing of the DLK1-Dio3 locus (Gtl2off) did not contribute well to chimaeras or form animals from tetraploid complementation experiments (all-iPSC mice). On the other hand, iPSCs with proficient expression from this locus (Gtl2on)—similar to that observed in their ESC counterpart—generated high-quality chimaeras and, more importantly, generated all-iPSC mice on tetraploid complementation. These observations may be a consequence of incomplete reprogramming, as suggested by a rescue experiment in which iPSC-Gtl2off lines were treated with the histone deacetylase inhibitor, valproic acid (VPA). Treatment coincided with reactivation of this locus and consequent generation of all-iPSC mice on tetraploid complementation. In addition, Gtl2 was highly expressed in fibroblasts from which the iPSCs were generated, whereas iPSCs derived from haematopoietic cell lineages which expressed low levels of Gtl2 consistently generated iPSCs with a Gtl2off status. The authors speculate that this may well be a consequence of epigenetic memory.

## Transcriptome analysis, the algorithm makes the difference!

The global gene expression profiles of iPSCs and ESCs derived from murine and human origins were compared by [Chin et al. \(2009\)](#). On face value, iPSCs appeared to be similar to ESCs but as observed by [Stadtfield et al. \(2010\)](#), gene expression patterns that were specific to iPSCs emerged. This was irrespective of their origin or method of generation. Interestingly, two follow-up studies based on genome-wide transcriptional analysis of similar data sets described by [Chin et al. \(2009\)](#) came to different conclusions. First, [Guenther et al. \(2010\)](#) compared both global chromatin structure and gene expression profiles of a panel of hiPSCs and hESCs. Interestingly, they observed very little variation with respect to histone H3K4me3 and histone H3K27me3 modification in both hiPSCs and hESCs. In addition, this was reflected in gene expression profiles that again exhibited very few differences. Importantly, the observed differences did not discriminate iPSCs from ESCs as was observed in the study by [Chin et al. \(2009\)](#). However, [Newman and Cooper \(2010\)](#) arrived at a very different conclusion. They re-analysed micro-array data sets from seven different laboratories that interrogated some 17 hESC lines, 67 hiPSCs lines and 18 fibroblast lines. Their analysis uncovered a striking correlation between the expression profiles of the hiPSCs or hESCs and the laboratory of origin (Fig. 1). This suggests that much of the available data may be artefacts of laboratory origin



**Figure 1** Laboratory-specific differences rendered as a fuzzy cluster network. Reprinted from Newman and Cooper (2010) with permission from Elsevier. In short, whole transcriptome analysis of human induced pluripotent cells (iPSC), embryonic stem cells (ESC) and fibroblast lines, illustrating distinct laboratory-specific gene expression signatures of PSCs. This figure demonstrates that individual laboratory-specific lines (both iPSC and ESC) cluster. In some cases, the transcriptional profiles of different laboratories are clustered (Daley, Sakurada and Lanza/Kim), which may reflect laboratory derivation and stem cell culturing practices.

and reinforces the importance of following best practice in experimental design and data analysis (Chin et al., 2010). Rigid standard operating procedures are required to reveal meaningful differences in cellular properties, as discussed by Loh and Lim (2010). Thus, it appears that culture conditions and the way the cell lines are handled may generate a particular laboratory signature on top of which the mode of reprogramming can still affect the end product (Chin et al., 2010).

## These differences are not restricted to iPSCs...

This phenomenon of subtle differences is not restricted to iPSCs versus ESCs. Abeyta et al. (2004) investigated a number of hESC lines and discovered that individual lines expressed unique gene signatures. A number of more recent studies demonstrate that ESCs exhibit variation in terms of their growth profiles and differentiation potential. Some lines exhibit a propensity to differentiate towards specific lineages, which is often accompanied with elevated lineage-specific gene expression (Osafune et al., 2008) and overall differential gene expression (Allegrucci and Young, 2007; Sharova et al., 2007; Tavakoli et al., 2009).

Aberrant imprinting of iPSCs, as referred to above (Stadtfeld et al., 2010), appears not to be unique to iPSCs. It has also been reported in other pluripotent cells types, notably EpiSCs derived from NT-blastocysts (Maruotti et al., 2010). Although EpiSC could be isolated with a similar efficiency from both fertilized and NT-blastocysts,

a striking difference was observed with respect to the gene expression profiles, with one-third of these differences being located on chromosome 11. In most cases, the genes imprinted were methylated on both alleles, and, as a consequence, their expression was down-regulated in cells derived from NT-blastocysts compared with counterparts from fertilized blastocysts.

## Conclusion

Current methods of observation and analysis reveal that iPSCs, ESCs and NT-ESCs are not absolutely equivalent with respect to epigenetic signature, differential potential or gene expression (Table I) (Chin et al., 2009; Kim et al., 2010; Maruotti et al., 2010; Stadtfeld et al., 2010). More importantly, it appears that iPSCs and ESCs in isolation are themselves are not equivalent, despite sharing all the prerequisites of pluripotency and capacity to self-renew. The reported transcriptional or epigenetic differences between iPSCs and ESCs therefore imply redundancies in the pluripotency network that have no overall significant impact on stem-cell pluripotency *per se*. These subtle differences may well be compounded by culture conditions and laboratory practices as exemplified by the work of Newman and Cooper (2010). Therefore, these differences could potentially be eradicated or minimized (managed) by integrating standard operating procedures with respect to the handling of ESC and iPSC lines with respect to culture conditions such as medium and extracellular matrices (Table II).

**Table 1** Overview of similarities and differences observed in PSC populations.

	ESCs	hESCs	iPSCs	hiPSCs	EpiSCs	NT-EpiSCs	Somatic cell
Properties							
Pluripotency and self-renewal	Yes	Yes	Yes	Yes	Yes	Yes	No
LIF/BMP4 dependent	Yes	No	Yes	No	No	No	ND
FGF2/Activin dependent	No	Yes	No	Yes	Yes	Yes	ND
Chimaera and 4N generation	Yes	ND	Contribute to chimaeras dependent on epigenetic status	ND	ND	Corrected by deletion of Xist, this gives increased efficiency of cloned live animals (Inoue <i>et al.</i> , 2010).	No
			4N animals—only when DLK1-Dio3 locus active (Gtl2on)			4N—ND	
Transcriptome	Unique signatures observed (Sharova <i>et al.</i> , 2007)	Unique signatures observed (Abeyata <i>et al.</i> , 2004; Allegrucci and Young, 2007)	Very similar to ESCs but differences observed (Stadtfield <i>et al.</i> , 2010)	Unique miR signatures observed by Malchenko <i>et al.</i> (2010), Chin <i>et al.</i> (2009), Wilson <i>et al.</i> (2009)	Very similar to ESCs	See below: transcriptional differences as a consequence of aberrant epigenetic profiles (Maruotti <i>et al.</i> , 2010)	Unique signatures—tissue specific
				Transcriptional similar to hESCs (Chin <i>et al.</i> , 2009, 2010; Guenther <i>et al.</i> , 2010). Newman and Cooper (2010) demonstrate that expression patterns laboratory specific			
Aberrant epigenetic profiles	ND	ND	Epigenetic memory of somatic origin early passage (Kim <i>et al.</i> , 2010; Polo <i>et al.</i> , 2010). Epigenetic memory removed with extended passaging or treatment with small molecules (as suggested by Stadtfield <i>et al.</i> , 2010) rendering functionally equivalent to ESCs	Epigenetic memory of somatic origin early passage (Marchetto <i>et al.</i> , 2009; Ghosh <i>et al.</i> , 2010)	Aberrant Xist expression can be corrected, thus generating live animals (Inoue <i>et al.</i> , 2010)	Many differences imprinting/epigenetic 30% on chromosome 11. (Maruotti <i>et al.</i> , 2010)	No

ESCs, embryonic stem cells; hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; hiPSCs, human induced pluripotent stem cells; EpiSCs, epiblast-derived stem cells (post-implantation); NT-EpiSCs, epiblast-derived stem cells (post-implantation) derived from somatic cell nuclear transfer; red indicates human source; blue indicates mouse source; purple indicates mouse epiblast source; ND, not described; LIF, leukaemia inhibitory factor; BMP4, bone morphogenic factor; FGF2, fibroblast growth factor 2.



**Table II** Summary of ‘manageable’ factors which influence epigenetic regulation of pluripotency and the practical implications. VPA, valproic acid; ESCs, embryonic stem cells.

Factors influencing epigenetic regulation of pluripotency	Practical implications
Tissue origin	Accessibility of donor tissue, potential donor tissue memory could influence differentiation potential (pros and cons)
Culture methods, medium and extracellular matrices	Standardized methods and reagents could potentially minimize observed differences
Extended passaging, small molecules, e.g. VPA	Ability to mirror ‘gold standard’ ESCs
Laboratory practices	Importance for protocols for stem cell research, diagnostics or therapies

Incomplete reprogramming of iPSCs is probably a consequence of the process being stochastic (Li *et al.*, 2009; Utikal *et al.*, 2009). The reprogramming process requires only four factors and it takes around a month to generate ES-like colonies. It is apparent from the work of Stadtfeld *et al.* (2010) that only a small percentage of generated pluripotent iPSCs are similar to their ESC counterparts in that they can contribute to a live animal in tetraploid complementation.

Getting back to the crux, are iPSC cells functionally equivalent to ESCs? Kim *et al.* (2010), Chin *et al.* (2009) and Polo *et al.* (2010) demonstrated that iPSCs, in early passage cultures, have a residual epigenetic memory of the tissue from which they were derived. In addition, variation both at the epigenetic level and at the transcriptional level occurs in other pluripotent cell populations, as discussed above. Yet, importantly, all these cell types share the common features of pluripotency and the ability to self-renew indefinitely. iPSCs generated using present-day methods appear to be very similar to ESCs with the above subtle differences, so there is still room for improvement. Stadtfeld *et al.* (2010) demonstrated that signatures of previous identity can be erased with relative ease and an ESC identity re-affirmed by the use of small molecules such as VPA. In addition, simply extended passaging causes amnesia within iPSCs! These observations may be indicative of a passive mechanism of epigenetic re-assertion. Very recently, Inoue *et al.* (2010) have demonstrated that deletion of Xist on the active X-chromosome restored normal global gene expression and resulted in increased cloning efficiency. Thus, it is apparent that we do not fully understand the mechanisms responsible for cellular re-assignment and it will require further investigation and refinement if we are to do so. To understand the implications of epigenetic memory, we need to consider the intended use of the reprogrammed cell population in question and establish a gold standard cell against which to assess it. Finally, other areas of reprogramming are rapidly evolving, such as trans-differentiation of one somatic cell type to another by forced expression of key transcription factors (Ieda *et al.*, 2010; Vierbuchen *et al.*, 2010). When it comes to assessing their practical benefits, the same concerns will apply.

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